

***Anti-Listeria monocytogenes* Activity of Human Neutrophils**

An Honors Research Thesis

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Abstract

Listeria monocytogenes is a facultative intracellular pathogen, which invades and replicates within a variety of cells, causing a severe foodborne illness in humans with a high fatality rate of 30%. The major virulence factor of *L. monocytogenes* is listeriolysin O (LLO), a secreted pore-forming toxin that perforates the phagosomal membrane and mediates bacterial escape to the cytoplasm, where replication occurs. Neutrophils and macrophages are professional phagocytes that are essential to the innate immune defense. They ingest microorganisms into phagosomes, which collect antimicrobial molecules to rapidly and efficiently kill microorganisms. It is well known that *L. monocytogenes* escapes from the phagosome in macrophages, avoiding their killing mechanisms and allowing the bacteria to multiply in the cytoplasm. However, little is known about the interactions of *L. monocytogenes* with neutrophils.

The aim of my project was to determine whether neutrophils, isolated from human peripheral blood, are able to ingest and kill *L. monocytogenes*, or whether LLO can defeat the neutrophil's antimicrobial mechanisms. This was accomplished by monitoring the efficiencies of wild type and LLO-deficient strains of *L. monocytogenes* phagocytosis and killing by neutrophils in comparison to that of macrophages (cell line RAW 264.7). Fluorescence-based microscopy methods were used to monitor phagocytic uptake and intracellular proliferation of bacteria. Intracellular and total (extracellular and intracellular) killing was measured using a gentamicin survival assay. Future directions include analysis of bacterial escape from the phagosome and/or fusion of neutrophil granules with the phagosome using immunofluorescence microscopy.

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1. Introduction

a. *Listeria monocytogenes* and listeriosis

Listeria monocytogenes is a Gram-positive pathogenic bacterium responsible for listeriosis, a severe food-borne illness in humans. Listeriosis is recognized as an important public health problem by the U.S. Centers for Disease Control and Prevention because of its high fatality rate of 30% (Listeriosis: General Information, 2011). Listeriosis is characterized by a large spectrum of clinical manifestations ranging from gastroenteritis to life-threatening meningoencephalitis and sepsis. The severity of listeriosis is due to the ability of *L. monocytogenes* to cross the intestinal, blood-brain, and placental barriers. Most severely affected are immunocompromised individuals, the elderly, infants, pregnant women, HIV-positive individuals, and cancer patients. The disease is diagnosed when *L. monocytogenes* is isolated from normally sterile fluids such as the blood or cerebrospinal fluid.

b. The *L. monocytogenes* intracellular life cycle

L. monocytogenes is a facultative intracellular pathogen, which invades and replicates within a variety of host cells to cause disease. Once internalized, the secreted pore-forming proteinaceous toxin, listeriolysin O (LLO), perforates the phagosomal membrane and mediates bacterial escape from the phagosome to the cytoplasm, where bacterial replication occurs (Figure 1). In the cytoplasm, *L. monocytogenes* polymerizes the host's actin cytoskeleton at one bacterial pole, which propels the bacterium randomly through the cytoplasm. When bacteria reach the cell periphery, the actin-based movement leads to the formation of extracellular protrusions that are taken up by adjacent cells. The bacteria are then entrapped within a double-membrane vacuole, from which they use LLO to escape. The cell-to-cell spreading of *L. monocytogenes* allows for efficient propagation of infection within tissues (Seveau, 2007; Cossart, 2011).

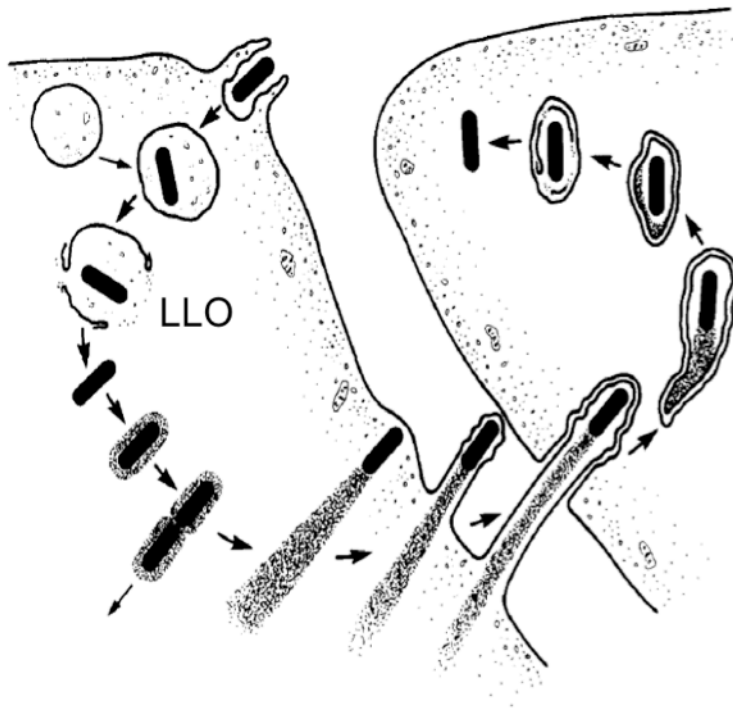


Figure 1: *L. monocytogenes* escapes from the phagosome using LLO and propagates infection (Tilney & Portnoy, 1989).

c. *L. monocytogenes* and neutrophils

Although it is well known that *L. monocytogenes* can survive in human and mouse primary macrophages and macrophage-like cell lines, the intracellular fate of *L. monocytogenes* in neutrophils is unclear. Neutrophils are short-lived but essential to the innate immune response, and rapidly and massively migrate from the blood to infected tissues in order to combat microorganisms during the first stages of an infection. These cells are professional phagocytes that ingest microorganisms into phagosomes. Unlike macrophages, neutrophils contain granules, which are intracellular compartments storing antimicrobial molecules. Neutrophil granules are able to fuse with the phagosome in order to exert potent antimicrobial activity. Neutrophils are also able to degranulate in the extracellular compartment, and in doing so they release antimicrobial molecules that may directly target extracellular pathogens. Three types of granules exist and each is distinguished based on specific contents (in parenthesis): primary (defensins, myeloperoxidase), secondary (lactoferrin), and tertiary (gelatinase). They also differ in their site of exocytosis. Secondary and tertiary granules are primarily released into the extracellular

environment whereas primary granules are primarily delivered to the phagosome (Borregaard, 2010).

Reports on neutrophil interaction with *L. monocytogenes* have been controversial. It has been shown that neutrophils are able to kill *L. monocytogenes*, but further studies reported that *L. monocytogenes* survives within neutrophils (Rowan, *et al.*, 2009). *In vivo* studies have shown that neutrophils exert a critical role in the control of *L. monocytogenes*, as the absence of neutrophils in animals and humans leads to exacerbated infections (Valdez *et al.*, 2009; Gregory & Wing, 2002; Unanue, 1997; Conlan & North, 1994; Czuprynski *et al.*, 1994; Rogers & Unanue, 1993).

2. Objectives of the Study

The aim of this study was to determine whether neutrophils, isolated from human peripheral blood, are able to ingest and kill *L. monocytogenes*, or whether the pore-forming toxin LLO is able to mediate *L. monocytogenes* escape from the neutrophil's phagosome, as seen in macrophages. We hypothesized that neutrophils would internalize and efficiently kill *L. monocytogenes* within the phagosome via fusion with the granules, because the granules contain high concentrations of antimicrobial molecules, such as defensins (Arnett & Seveau, 2011). Our laboratory has shown that defensins are potent anti-LLO proteins that prevent *L. monocytogenes* phagosomal escape in macrophages pre-loaded with synthetic defensins (Arnett *et al.*, 2011). Macrophages lack defensins, and therefore we expected neutrophils to be more efficient than macrophages in preventing phagosomal escape. We also hypothesized, based on the literature, that neutrophils could kill extracellular bacteria. Indeed, neutrophils are able to release the contents of their granules and reactive oxygen species (ROS) into the extracellular environment (Faurschou & Borregaard, 2003).

This study measured the neutrophil's phagocytotic efficiency, and the intracellular survival of *L. monocytogenes* in neutrophils infected with various multiplicities of infection. Our study also examined possible evidence of a neutrophil extracellular killing mechanism.

3. Methods

a. Acquisition and culture of human neutrophils and macrophage cell line

Glass coverslips were coated with 100 µg/mL human fibronectin for 1 hour the night before the experiment. Human peripheral blood was obtained from healthy adult volunteers on the morning of the experiment using the venipuncture method, as approved by The Ohio State University Office of Research. Heparin tubes were used for blood collection and serum separator tubes with thrombin were used for serum collection. Neutrophils were isolated using Axis-Shield Polymorphprep density gradient centrifugation method (Polymorphprep). Blood tubes were inverted 10 times immediately after collection, and serum tubes sat for 15 min before centrifugation. Blood and serum were centrifuged at 900 rotations per minute (rpm) for 15 min at room temperature without the use of a brake. Serum was removed from the serum tubes, and the complement system was inactivated at 56°C for 30 min. Plasma was removed from the blood tubes, the volume was replaced with Hank's Balanced Salt Solution without calcium or magnesium (HBSS-), in order to prevent neutrophil activation. The pellet was gently resuspended. Polymorphprep (3.5 mL) was added to 15 mL tubes, and 5 mL blood was added to the top of each tube. Tubes were centrifuged at 1500 rpm for 35 min at room temperature with no brake. Layers of peripheral blood mononuclear cells (PBMC) and plasma were removed, and neutrophils were collected (Figure 2).

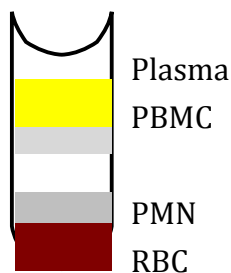


Figure 2: Density layers following centrifugation with Polymorphprep: blood plasma, peripheral blood mononuclear cells (PBMC), polymorphonuclear neutrophils (PMN), and red blood cells (RBC).

Neutrophils were washed in HBSS- and centrifuged at 1400 rpm for 10 min at room temperature with low brake. Pellet was aspirated and resuspended in about 1 mL.

Remaining red blood cells were lysed by hypotonic shock in sterile water for 30 seconds, and neutrophils were stored in HBSS-. Neutrophils (4.5×10^5 cells per well) in HBSS+ and 10% donor serum by volume were allowed to attach to the fibronectin-coated wells for 10 min.

Thp-1 macrophage cell line (ATCC Number TIB-202) was passaged every 2-3 days with a 1/10 dilution in Roswell Park Memorial Institute (RPMI) media with 100 units Pen/Strep and 10% heat inactivated Fetal Bovine Serum (HI-FBS). Forty-eight h before the experiment, cells were centrifuged at 1500 rpm for 5 min at room temperature. Media was replaced and cells were resuspended, counted, and seeded at 8×10^4 cells/well in order to achieve a final count of 7×10^4 cells/well due to lack of division after differentiation and an approximately 90% attachment rate. Cells were differentiated with 160nM PMA and incubated for the remaining 48 h.

RAW 264.7 macrophage cell line (ATCC Number TIB-71) was passaged every 2-3 days with a 1/10 dilution in Dulbecco's Modified Eagle Medium (DMEM) complemented with 100 units Pen/Strep and 10% HI-FBS. The cells were seeded 24 h before the experiment at half the desired concentration, in this case, 1.1×10^5 cells/well. This number was decreased from that of neutrophils, but is still comparable, due to a difference in attachment rate.

L. monocytogenes wild-type (wt) and Δhly were cultured overnight in 5 mL Brain Heart Infusion (BHI) media. The morning of the experiment, the bacterial cultures were diluted 1/20 and grown to optical density (OD) 0.7-0.8. Bacteria were washed in Phosphate Buffered Solution (PBS).

b. Measurement of the efficiency of neutrophil phagocytotic uptake

The efficiency of *L. monocytogenes* internalization by neutrophils and macrophages was measured by quantitative fluorescence microscopy using an assay developed in our laboratory (Haghighat, 2010). Neutrophils were isolated from human blood as described above, with one exception. The use of serum in the media was included as a variable, which allows opsonization of the bacteria. These experiments were performed both in HBSS without serum and HBSS+/ 10% donor serum. Neutrophils were incubated with *L. monocytogenes* wt or Δhly for 30 min at

MOI 10 or 1 (4.5×10^6 and 4.5×10^5 per well, respectively). Neutrophils were washed to remove extracellular unbound bacteria, and either fixed with 3.5% paraformaldehyde (PFA) in PBS with calcium and magnesium, or further incubated for 2.5 or 4.5 h in the presence of $15 \mu\text{g/mL}$ gentamicin. Gentamicin is a cell membrane-impermeant antibiotic that kills extracellular bacteria. After a total incubation period of 3 or 5 h, these neutrophils were fixed with PFA. Neutrophils were blocked for 45 min with 0.1 M glycine in PBS with 10% FBS. A primary anti-*Listeria* antibody was then used to label bacteria, in addition to a secondary antibody conjugated to a fluorochrome, Alexa488. This labels extracellular bacteria bound to the cell (Figure 3a). Cells were permeabilized for 2 min with 0.1% Triton X-100 in PBS, and the bacteria were labeled by the same procedure, but this time using a secondary fluorescent antibody conjugated to a second fluorochrome, Alexa568, to label the total bacterial population (extracellular and intracellular). An automated fluorescent microscope and associated computer software were used to acquire and analyze images. The amount of intracellular bacteria, used to characterize the phagocytosis of *L. monocytogenes* in neutrophils, was obtained from subtraction of bacteria labeled with the first fluorochrome from the second (Figure 3b). These experiments were repeated with macrophages (cell line Thp-1) in RPMI media without serum for comparison.

Figure 3a: Extracellular bacterial population with fluorescent labeling, with intracellular unlabeled bacteria circled.

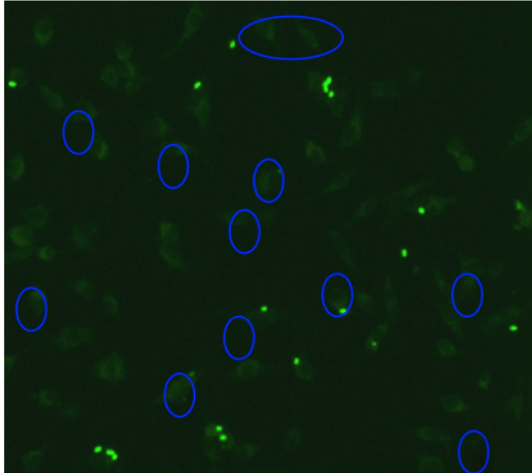
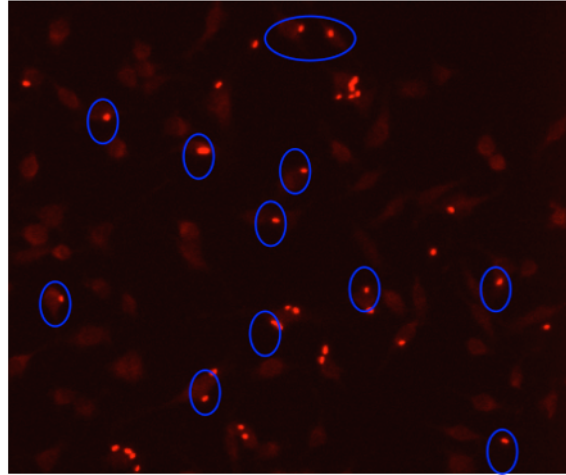


Figure 3b: Total bacterial population with fluorescent labeling, with intracellular bacteria circled.



c. Measurement of *L. monocytogenes* survival in the presence of neutrophils

Cell culture wells on a 24-well dish were coated with 100 $\mu\text{g}/\text{mL}$ human fibronectin for 1 hour the night before the experiment. Neutrophils were isolated from human blood and plated in HBSS with 10% donor serum as described above. *L. monocytogenes* wt or Δhly were added at MOI 10 or 1, (4.5×10^6 and 4.5×10^5 per well, respectively), and incubated for 1.5 or 5 h. Following this incubation, neutrophils were lysed with 0.2% Triton X-100, and the lysates were plated on BHI agar to enumerate the total (intracellular and extracellular) surviving bacteria. A second set of wells was treated as above, with the addition of 15 $\mu\text{g}/\text{mL}$ gentamicin for the last hour. Following this incubation, neutrophils were washed to remove the gentamicin, and lysed. The lysates were plated on BHI agar to enumerate the intracellular surviving bacteria. These same conditions were repeated, with the exception of cell culture media which this time was DMEM with 10% donor serum, for assays using a 5 hour incubation with macrophages (cell line RAW 264.7) for comparison. Three independent experiments were performed in duplicate for both neutrophils and macrophages. T-tests were performed to determine statistical significance.

d. Characterization of a neutrophil extracellular killing mechanism

A two-compartment transwell setup was used to determine whether neutrophils employed an extracellular contact-independent killing mechanism (Figure 4). Top compartments were separated from the bottom compartments with a 0.4 μ m filter, and were in contact through solution only (*Corning Transwell Permeable Supports*, 2011). Neutrophils (4.5×10^5 per well) in HBSS+ with 10% donor serum were allowed to attach to fibronectin-coated plastic in the bottom compartment of the wells for 10 min. Neutrophils were i) activated with *L. monocytogenes* wt or Δhly at MOI 10 (4.5×10^6 per well), ii) chemically induced to degranulate with 1 μ M latrunculin A for 30 min and 300nM f-met-leu-phe (f-MLP) added for the last 5 min of that incubation, or iii) left inactivated. Control bottom compartments contained i) the cell culture media without neutrophils, ii) *L. monocytogenes* wt or Δhly alone at MOI 10, iii) degranulation induction chemicals alone, or iv) *L. monocytogenes* wt at 6×10^4 bacteria/well. Top compartments of each well received *L. monocytogenes* wt at an equivalent of MOI 1 (4.5×10^5 per compartment). The plate was incubated for 5 h, and bacteria above the filter were enumerated on BHI agar. T-tests were performed to determine statistical significance.

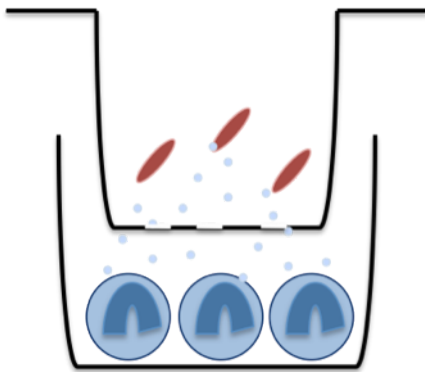


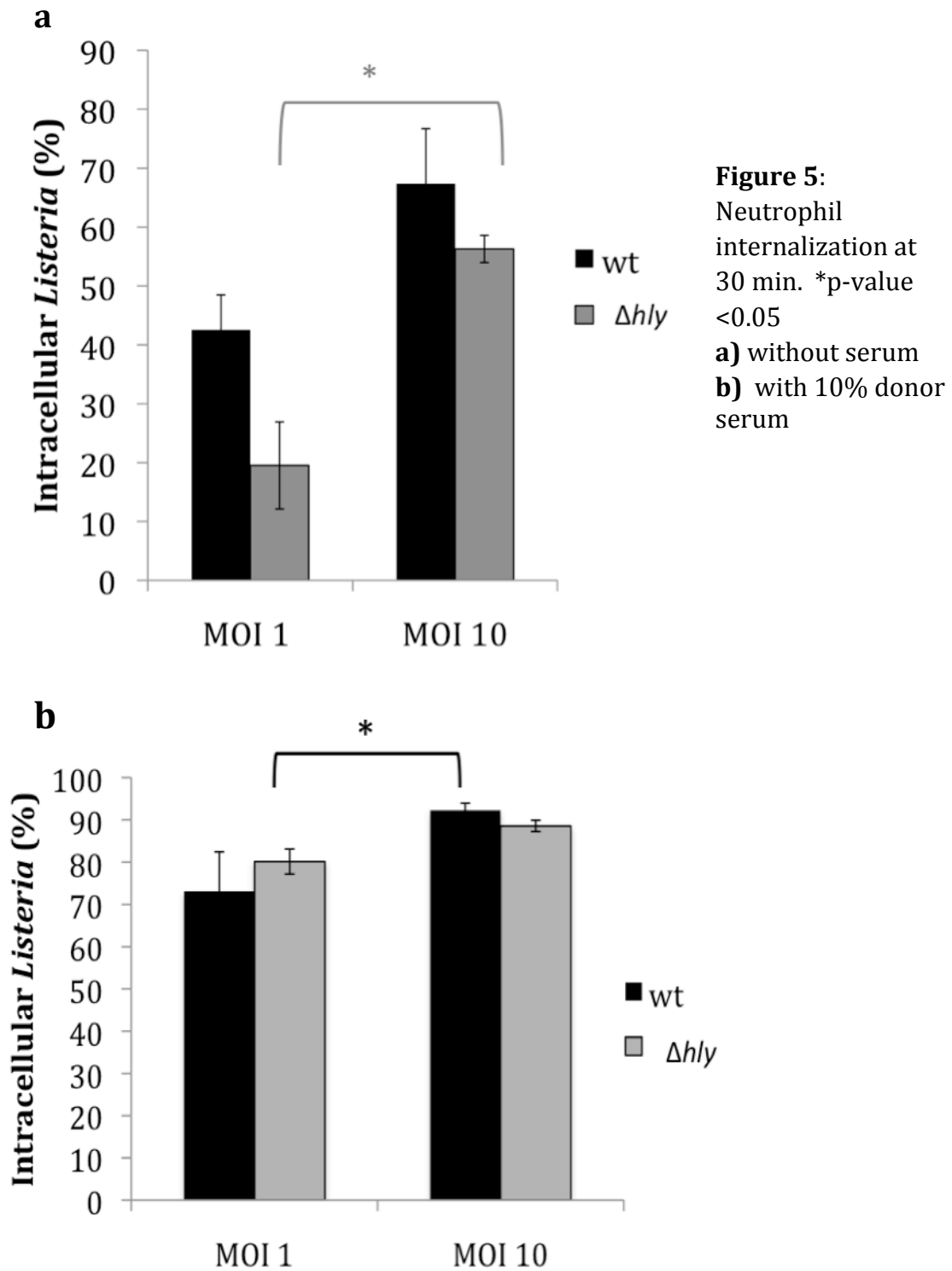
Figure 4: Two compartment transwell setup. *L. monocytogenes* wt added to the top compartment with varied conditions in the bottom compartment.

4. Results

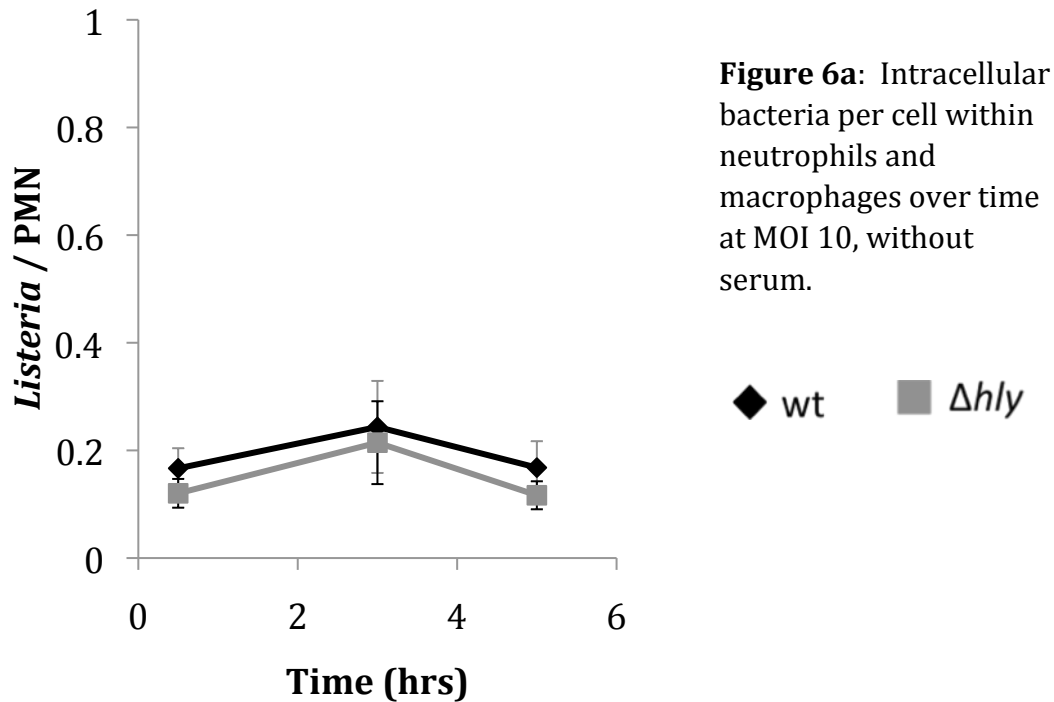
a. Neutrophils efficiently phagocytose *L. monocytogenes* and prevent intracellular growth

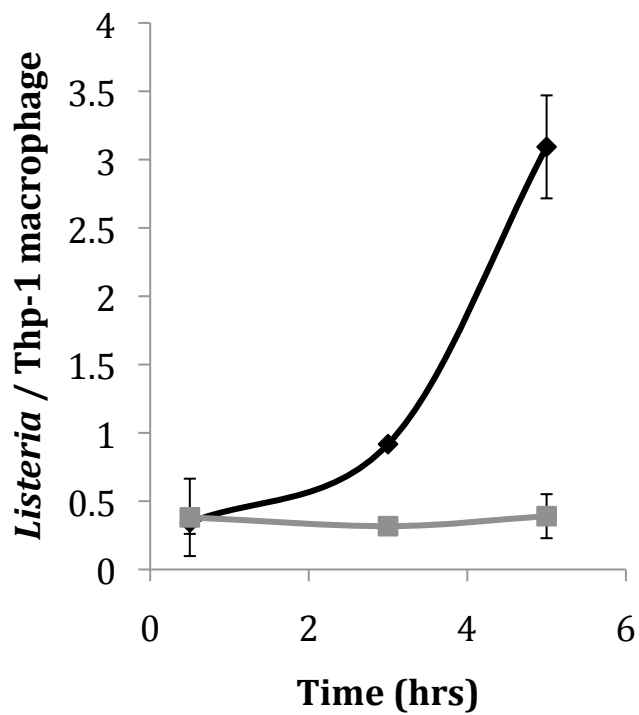
At 30 min without serum, about 60% of the neutrophil-associated bacteria were intracellular when infected at MOI 10 and about 30% were intracellular when infected at MOI 1 (Figure 5a). This figure indicates that neutrophils do phagocytose *L. monocytogenes* without opsonization, though more efficiently when infected at MOI 10, indicating that the presence of bacteria activates neutrophil phagocytic activity. T-tests were performed and there appears to be a significant difference in internalization between the *L. monocytogenes* Δhly at MOI 10 and 1, but the difference between strains is not significant.

In the presence of serum, the large majority of neutrophil-associated bacteria were intracellular after 30 min. There is a slight difference in the percent intracellular bacteria at MOI 1, which is about 80%, and MOI 10, which is about 90% (Figure 5b). After T-Tests were performed, the only significant difference was between *L. monocytogenes* Δhly MOI 1 and *L. monocytogenes* wt MOI 10. These results indicate that bacterial opsonization by serum components, likely IgGs, increases *L. monocytogenes* uptake, or that the serum directly activates neutrophil phagocytic activity.



Over the course of 5 h following bacterial uptake for 30min in the absence of serum, we observed a constant bacteria to neutrophil ratio around 0.2 for both *L. monocytogenes* wt and Δhly (Figure 6a). This result indicates that the number of bacteria per neutrophil remains constant; therefore, bacteria are likely unable to multiply within neutrophils. In macrophages, this ratio remains approximately constant for *L. monocytogenes* Δhly but the ratio for *L. monocytogenes* wt steadily increased over time. This difference in bacteria to macrophage ratio is likely due to the ability of *L. monocytogenes* wt to escape the phagosome and replicate intracellularly, whereas *L. monocytogenes* Δhly remains entrapped within the phagosome and is not able to replicate.





When bacterial uptake occurred in the presence of serum, phagocytosis was more efficient, leading to an initial bacterium to neutrophil ratio of 4. Again, we found that this ratio is constant during the experiment (Figure 6b). The neutrophils were able to exert a similar effect on both bacterial strains, indicating a lack of protective effect of LLO.

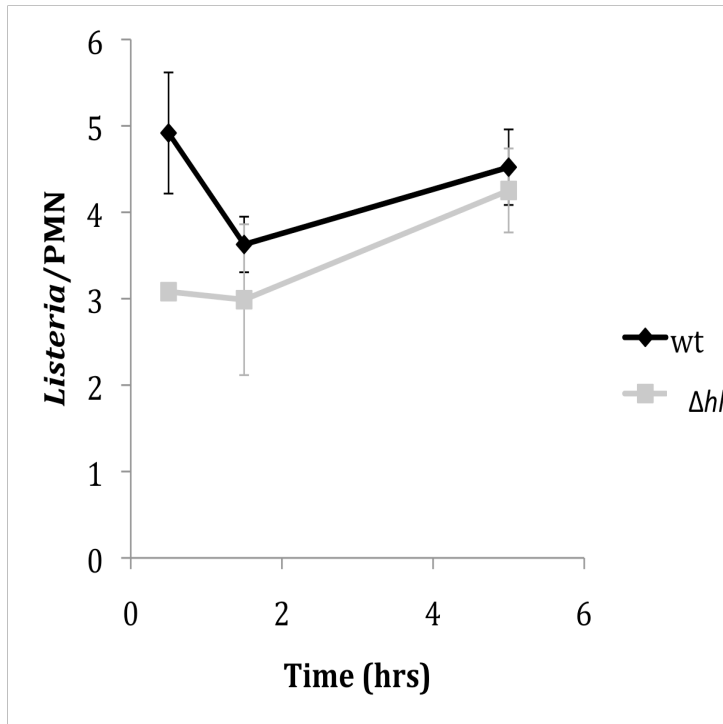
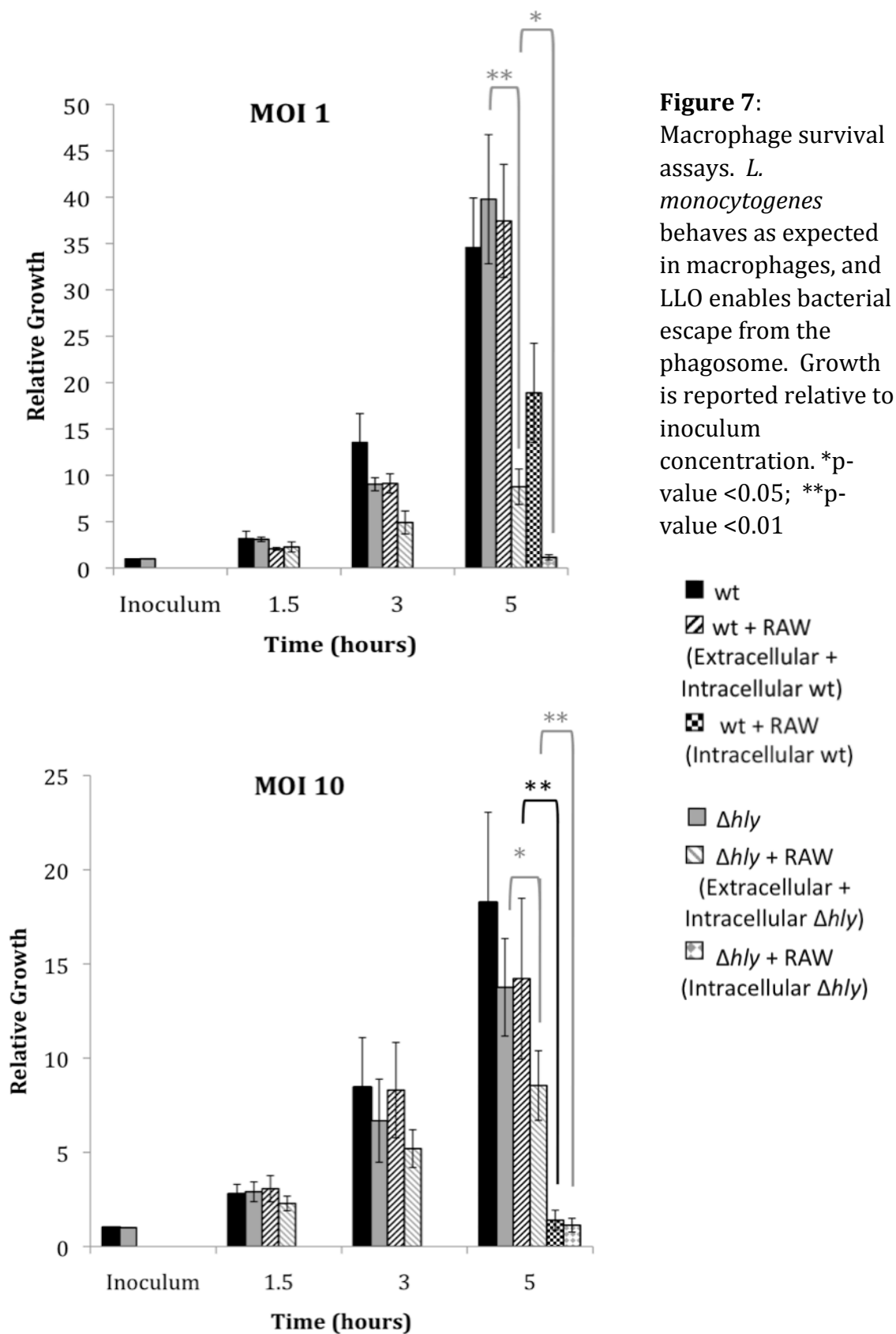


Figure 6b:
Intracellular bacteria
per cell within
neutrophils over time
at MOI 10, with 10%
donor serum.

b. Neutrophils greatly reduce the survival of *L. monocytogenes*

The results of the survival assays show that in the presence of macrophages, *L. monocytogenes* behaves as expected. Over the course of 5 h, *L. monocytogenes* wt grew at a similar rate in the presence or absence of macrophages (Figure 7). At MOI 10, macrophages were visibly damaged by *L. monocytogenes* wt as assessed under a light microscope. This damage was due to the intracellular multiplication of the bacteria.

L. monocytogenes Δhly succumbs to the macrophage's killing mechanisms and a diminished overall growth rate was observed. Bacteria were still able to grow past the inoculum concentration, but did not grow as efficiently as in the cell culture media alone. This is likely due to the lack of secretion of LLO and the inability of *L. monocytogenes* Δhly to escape from the phagosome. Intracellularly, *L. monocytogenes* Δhly barely moves beyond the inoculum concentration. This is evidence for successful killing within the macrophage phagosome.



In the presence of neutrophils, *L. monocytogenes* behaves differently. Neutrophils have a significant inhibitory effect on the growth of *L. monocytogenes*, and the difference in growth between the wt and Δhly strains was insignificant (Figure 8). At MOI 10, neutrophils were able to produce a 5-fold reduction at 5 h as compared to normal growth in the cell culture media. At MOI 1, this figure jumps to a 31-fold reduction in growth. Intracellular survival in neutrophils at 5 h accounted for less than 1% of the total survival, which means that the large majority of surviving bacteria at 5 h were extracellular.

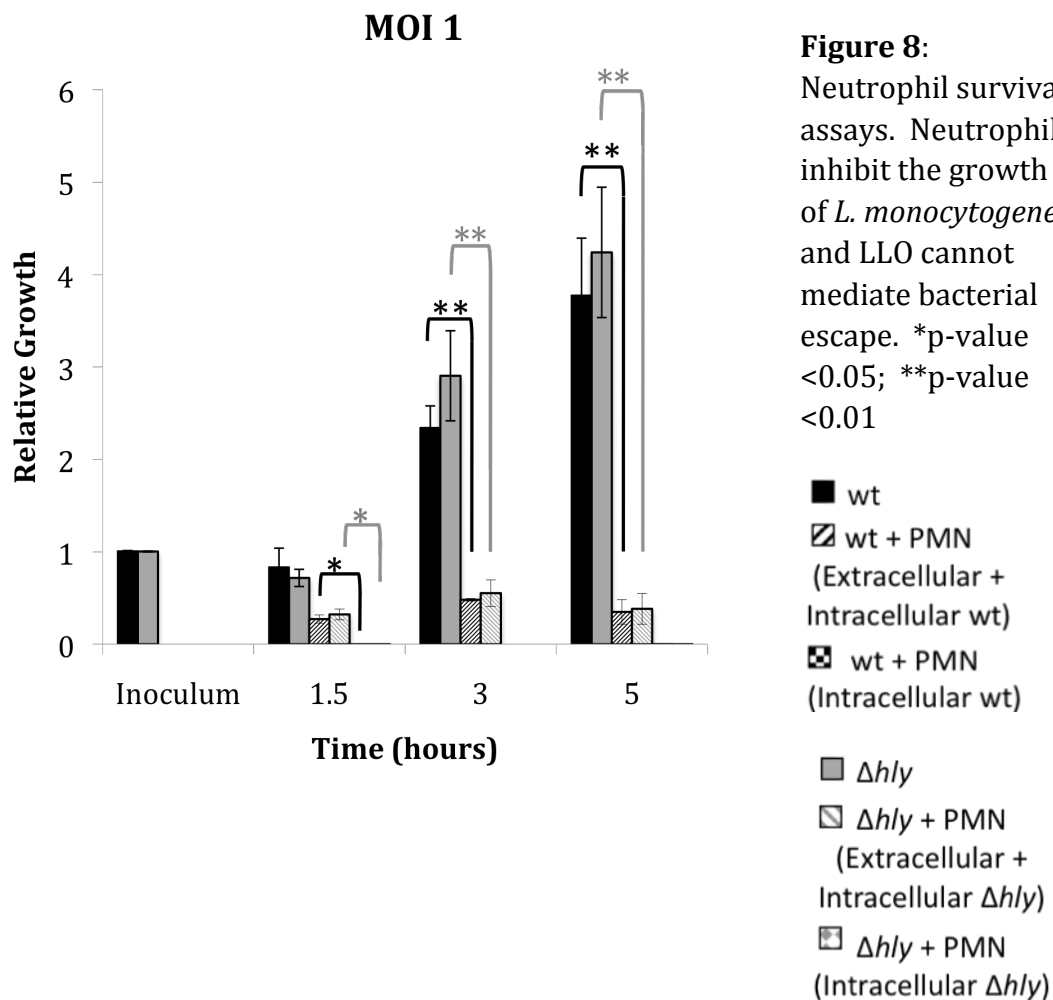
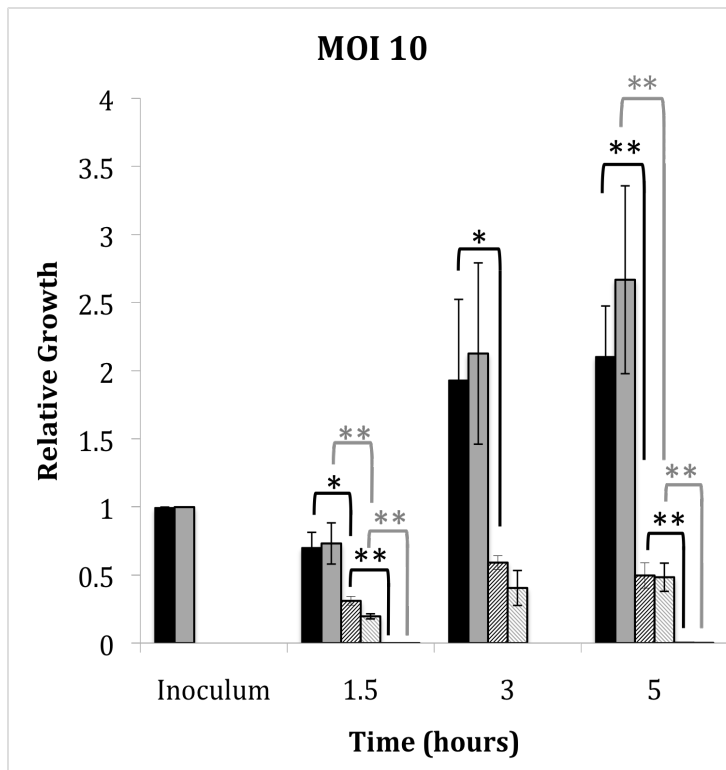


Figure 8: Neutrophil survival assays. Neutrophils inhibit the growth of *L. monocytogenes* and LLO cannot mediate bacterial escape. *p-value <0.05; **p-value <0.01

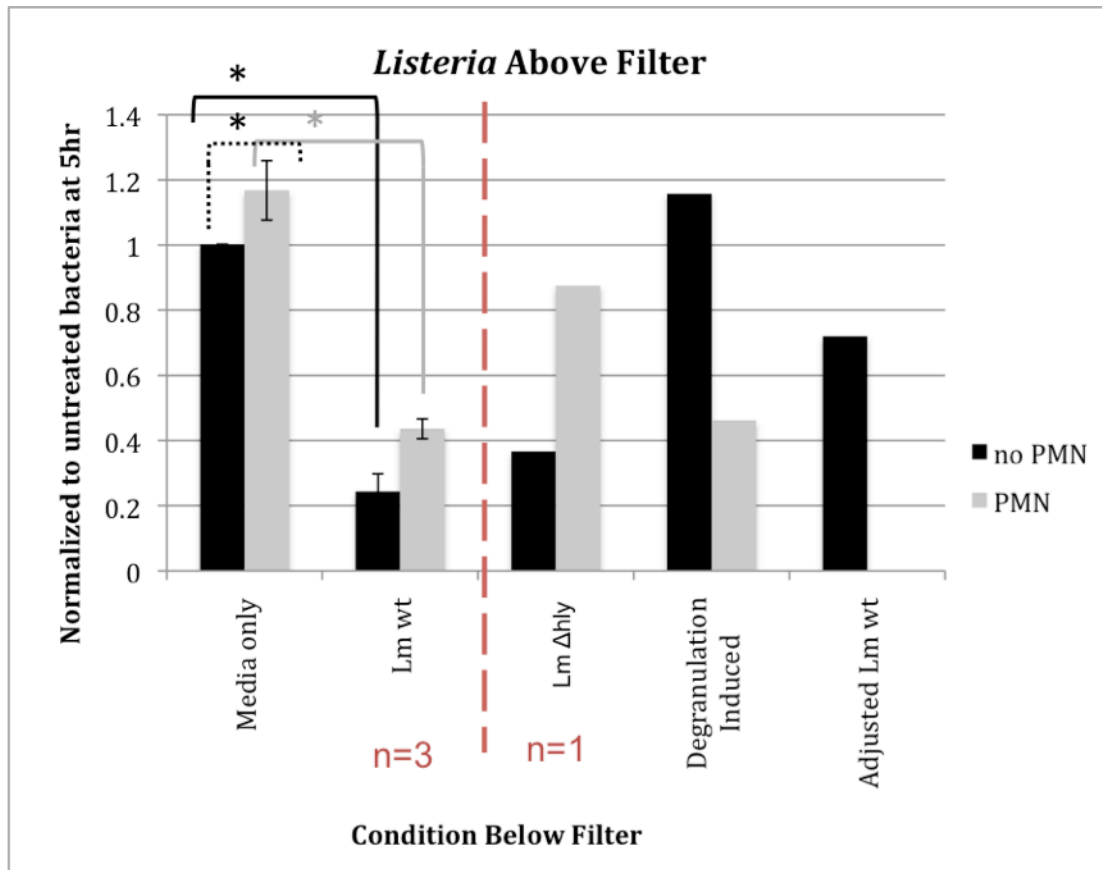


c. Neutrophils may employ an extracellular contact-independent killing mechanism

Neutrophils co-incubated with *L. monocytogenes* wt at MOI 10 significantly decreased the growth of *L. monocytogenes* in the upper compartment (Figure 9). Preliminary results for neutrophils chemically induced to degranulate showed a similar growth inhibition effect, and degranulation treatment alone without neutrophils had no difference in growth. If co-incubated with *L. monocytogenes* Δhly , neutrophils showed less of an effect on the growth of bacteria in the upper compartment. However, to our surprise, the bacteria alone in the bottom compartment exerted a negative effect on the growth of the bacteria in the top compartment, in the absence of neutrophils. “Adjusted *Lm* wt” is less than MOI 10 in order to mimic the decrease in bacteria that would have occurred had the neutrophils been present. Preliminary results show an effect that is not as large as in the presence of neutrophils, but it is still likely significant. Upper compartment bacteria in contact with a lower compartment of inactivated neutrophils grow

slightly better than those in contact with a lower compartment of cell culture media alone.

Figure 9: Transwell assays. Neutrophils activated by *L. monocytogenes* wt or induced to degranulate release soluble components that have antibacterial activity. *p-value <0.05.



5. Discussion

The results of the immunofluorescence internalization assays indicate that neutrophils are able to efficiently phagocytose *L. monocytogenes* within 30 min. At MOI 10 in the absence of serum, *L. monocytogenes* wt is internalized more efficiently than *L. monocytogenes* Δhly , suggesting that LLO facilitates neutrophil internalization (Figure 5). This effect has also been observed in nonphagocytic cells (Vadia et. al., 2011), however, the mechanism used by LLO to affect bacterial uptake in neutrophils might be unrelated to the role of LLO in bacterial uptake by nonphagocytic cells. In the presence of serum, this difference is less pronounced.

LLO does not have an effect on the bacteria to cell ratio over time in neutrophils as it does in macrophages (Figure 6a). In macrophages, *L. monocytogenes* is able to use LLO to perforate the phagosome and escape to the cytoplasm, where it then replicates and increases the bacteria to cell ratio. This may be evidence of a difference in phagosomal environment between neutrophils and macrophages. It has been shown that LLO is more active at acidic pH than neutral (Glomski et. al., 2002). After phagocytosis, macrophage phagosomes acidify to pH 4-5. In neutrophil phagosomes, the pH remains around pH 7 (Nordenfelt & Tapper, 2011). In addition, neutrophil primary granules deliver high concentrations of defensins to the phagosome, and defensins are known to exert anti-LLO activity. The neutrophil's inhibitory effect on intracellular growth could either be due to intracellular killing or the exertion of a bacteriostatic effect. One limitation of the immunofluorescence method is the possibility of labeling dead bacteria. In this case, a constant bacteria to cell ratio may not mean a constant live bacterial population, but rather nonliving bacterial cells visualized inside the neutrophil. To determine whether neutrophils were exerting a bacteriostatic or bactericidal effect on *L. monocytogenes*, we then performed gentamicin survival assays. In addition to determining the viability of intracellular bacteria, this assay could test whether phagocytes kill extracellular bacteria.

We found that neutrophils markedly inhibited the growth of *L. monocytogenes* wt and efficiently killed all ingested bacteria, whereas these bacteria

were able to grow within macrophages, as expected (Figures 7, 8). Both neutrophils and macrophages impeded *L. monocytogenes* Δhly growth, although neutrophils exerted more efficient killing activity.

We next determined whether neutrophils could also kill extracellular bacteria. The transwell assay showed promising evidence of a neutrophil extracellular contact-independent killing mechanism. Though neutrophils activated by *L. monocytogenes* had an antibacterial effect on the top compartment bacteria, a similar trend was observed when the bottom compartment was occupied by *L. monocytogenes* alone. Further studies will be required to tease apart these effects. This difference may be due to nutrient competition between the two compartments or other processes, which leaves less significance for the activated neutrophil's effects. Nevertheless, as a proof of concept, induction of neutrophil degranulation by chemical treatment could efficiently inhibit *L. monocytogenes* growth by a contact-independent mechanism.

In conclusion, neutrophils efficiently phagocytose and kill intracellular *L. monocytogenes*, and LLO does not play a protective role for the bacteria in the presence of neutrophils. We believe that this difference between neutrophils and macrophages is likely due to a difference in phagosomal environment, as well as the neutrophil's combined ability to kill both intracellular and extracellular bacteria.

6. Future Directions

Future studies will focus on 1) elucidating the neutrophil's extracellular killing mechanism, 2) understanding why LLO is unable to mediate *L. monocytogenes* escape from the phagosome, and 3) determining the intracellular trafficking of *L. monocytogenes* in neutrophils.

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